Regulation of the Transient Outward K⁺ Current by Ca²⁺/Calmodulin-Dependent Protein Kinases II in Human Atrial Myocytes

Sophie Tessier, Peter Karczewski, Ernst-Georg Krause, Yves Pansard, Christophe Acar, Michel Lang-Lazdunski, Jean-Jacques Mercadier, Stéphane N. Hatem

Abstract—Ca²⁺/calmodulin-dependent protein kinases II (CaMKII) have important functions in regulating cardiac excitability and contractility. In the present study, we examined whether CaMKII regulated the transient outward K⁺ current (Iₒ) in whole-cell patch-clamped human atrial myocytes. We found that a specific CaMKII inhibitor, KN-93 (20 μmol/L), but not its inactive analog, KN-92, accelerated the inactivation of Iₒ (τᵢₒᵣᵨ, 66.9±4.4 versus 43.0±4.4 ms, n=35; P<0.0001) and inhibited its maintained component (at +60 mV, 4.9±0.4 versus 2.8±0.4 pA/pF, n=35; P<0.0001), leading to an increase in the extent of its inactivation. Similar effects were observed by dialyzing cells with a peptide corresponding to CaMKII residues 281 to 309 or with autacamtide-2–related inhibitory peptide and by external application of the calmodulin inhibitor calmidazolium, which also suppressed the effects of KN-93. Furthermore, the phosphatase inhibitor okadaic acid (500 nmol/L) slowed Iₒ inactivation, increased Iᵦᵨ, and inhibited the effects of KN-93. Changes in [Ca²⁺]ᵢ by dialyzing cells with ≈30 nmol/L Ca²⁺ or by using the fast Ca²⁺ buffer BAPTA had opposite effects on Iₒ. In BAPTA-loaded myocytes, Iₒ was less sensitive to KN-93. In myocytes from patients in chronic atrial fibrillation, characterized by a prominent Iᵦᵨ, KN-93 still increased the extent of inactivation of Iₒ. Western blot analysis of atrial samples showed that δ-CaMKII expression was enhanced during chronic atrial fibrillation. In conclusion, CaMKII control the extent of inactivation of Iₒ in human atrial myocytes, a process that could contribute to Iₒ alternations observed during chronic atrial fibrillation. (Circ Res. 1999;85:810-819.)

Key Words: KN-93 ■ K⁺ channel ■ δ-CaMKII ■ atrial fibrillation ■ heart

In human atrial myocytes, the transient outward K⁺ current (Iₒ) is essential for shaping and modulating the action potential. It is responsible for the early repolarization phase (“notch”) of the action potential and for the termination of the plateau phase. In addition, the frequency-dependent reactivation of Iₒ explains how this current plays a major role in the adaptation of the action potential duration and cellular refractory periods to changes in cardiac cycle lengths. In both diluted and fibrillating atria, alterations of Iₒ, together with a marked reduction in L-type Ca²⁺ current density, are responsible for the shortening of the cellular action potential and the poor frequency-dependent adaptation of the refractory period.¹² Both abnormalities favor the initiation and perpetuation of atrial arrhythmias. Studies of the mechanisms that regulate Iₒ are therefore crucial to understand the physiology and pathophysiology of the atrial myocardium.

The transient outward K⁺ current, recorded in isolated human atrial myocytes during a step depolarization, is composed of a rapidly inactivating component Iᵦᵨ and a sustained component Iᵦᵨ.³ This complex time course of Iₒ reflects the phenotypic diversity of K⁺ channels in cardiac myocytes. Indeed, a number of electrophysiological, pharmacological, and molecular observations indicate that Iₒ in human atrial myocytes is the functional expression of K⁺ channels with rapid N-type inactivation, whereas Iᵦᵨ is transported by slowly inactivating K⁺ channels such as hKv1.5 Shaker channels.⁴,⁵ which are abundantly expressed in human atrial myocardium.⁶,⁷ The characteristics of Iₒ activation and inactivation are also influenced by a variety of factors, including redox state⁸ and pharmacological agents.⁹,¹⁰ For instance, we found that the antiarrhythmic agent bertosamil can transform the noninactivating current Iᵦᵨ into a rapidly inactivating current by binding to an intracellular site.¹⁰ A number of intracellular regulatory pathways can also modulate Iₒ in human atrial myocytes. This is the case of β- and α-adrenergic pathways, which regulate Iᵦᵨ via cAMP-dependent protein kinases and protein kinases C, respectively.¹¹ It has also been proposed that the downregulation of Iᵦᵨ by atrial natriuretic peptide reflects the coupling between K⁺ channels and G protein.¹²

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810
Recent studies have suggested that Ca\(^{2+}/\)calmodulin-dependent protein kinases (CaMKII) modulate the inactivation of voltage-dependent K\(^+\) channels.\(^{13-15}\) For instance, CaMKII considerably slows the inactivation of Kv1.4 channels expressed in HEK-293 cells by phosphorylating a modulatory site located in the amino terminal cytoplasmic domain of these K\(^+\) channels. CaMKII are abundantly expressed in mammalian heart, \(\delta\)-CaMKII being the predominant isoform.\(^{16-19}\) These kinases have important functions in regulating cardiac myocyte excitability and contractility. For instance, CaMKII modulate the frequency and voltage facilitation of L-type Ca\(^{2+}\) channels in rat ventricular myocytes\(^{20}\) and the Ca\(^{2+}\)-induced enhancement of the L-type Ca\(^{2+}\) current in rabbit ventricular myocytes.\(^{21}\) Moreover, in pathophysiological conditions characterized by [Ca\(^{2+}\)]\(_d\), overload, CaMKII inhibition prevents the development of the arrhythmogenic transient inward current in rabbit ventricular myocytes.\(^{22}\) However, no data are available on the regulatory effect of CaMKII on K\(^+\) currents of cardiac myocytes, except for the identification of consensus sites for CaMKII phosphorylation on deduced amino acid sequences of several Kv channels expressed in heart.\(^{23,24}\)

The aim of the present study was to determine the contribution of CaMKII to the regulation of \(I_{\text{to}}\) activity in human atrial myocytes. Using whole-cell patch-clamp and immunocytochemistry techniques and various pharmacological agents, we obtained evidence that CaMKII are functionally coupled to \(I_{\text{to}}\) in human atrial myocytes, and that they regulate the rate and extent of inactivation of the current.

**Materials and Methods**

**Cardiac Myocyte Isolation**

Myocytes were enzymatically isolated from right atrial appendages obtained from 87 adult patients aged 15 to 84 years (mean 60.3 ± 1.7 years) undergoing heart surgery for coronary artery disease (n = 37), mitral valve disease (n = 23), aortic valve disease (n = 26), or tricuspid insufficiency (n = 5). Myocytes were isolated as previously described using collagenase (type IV, Sigma) and protease (type XXIV, Sigma).\(^{10}\) Currents were recorded using the patch-clamp technique in the whole-cell configuration. (Axoclamp 200A, Axon Instruments).

**Solutions and Drugs**

The external solution was composed of (in mmol/L) NaCl 137, KCl 5.4, CaCl\(_2\) 2, MgCl\(_2\) 1, HEPES 10, and glucose 10, adjusted to pH 7.3 with NaOH. For K\(^+\) current measurements, Na\(^+\) was replaced by an equimolar concentration of choline chloride, Ca\(^{2+}\) channels were blocked with 0.5 mmol/L Cd\(^{2+}\), and 10 \(^-4\) mol/L atropine was added to the external solution to prevent muscarinic receptor activation.

The internal solution contained (in mmol/L) potassium aspartate 115, MgATP 5, MgCl\(_2\) 3, EGTA 10, and HEPES 10, adjusted to pH 7.2 with KOH. In some experiments, EGTA was replaced by 40 mmol/L BAPTA. To test the effect of Ca\(^{2+}\) on \(I_{\text{to}}\), the following internal solution was used (in mmol/L): KCl 115, MgATP 5, NaCl 5, MgCl\(_2\) 3, EGTA 10, HEPES 10, and CaCl\(_2\) 1, adjusted to pH 7.2 with KOH, which yielded a free Ca\(^{2+}\) concentration of \(\sim\)30 mmol/L. All experiments were carried out at room temperature (22°C to 24°C).

KN-93, KN-92, and okadaic acid were from Calbiochem. Calmodulin was from Sigma. KN-93 was dissolved in DMSO, and the final solvent concentration was <0.05%, a concentration that had no effect on the outward K\(^+\) current. The Ca\(^{2+}\)/calmodulin kinase II inhibitors (peptide [AIP]; Calbiochem) were dissolved in the internal solution as well as BAPTA.

**Immunoblotting and Immunohistochemistry**

Ten micrograms of homogenate obtained from frozen atrial tissue was solubilized, boiled, and loaded on the top of 10% SDS polyacrylamide gels.\(^{25}\) The membranes were processed for immunoblotting as described elsewhere.\(^{26}\) \(\delta\)-CaMKII were detected with an antibody that specifically recognizes the C-terminal amino acid sequence unique to a subset of \(\delta\)-subunit variants.\(^{19}\) To correct for the amount of muscle protein in homogenates from individual tissue samples, the optical density (OD) values for \(\delta\)-CaMKII were calculated relative to OD values for myosin (205 kDa) obtained from Coomassie blue-stained blot membranes.

Indirect immunofluorescence was performed on 5-μm human atrium cryosections using the anti-\(\delta\)-CaMKII antibody (5 μg/mL).

**Data Analysis**

The time course of \(I_{\text{to}}\) inactivation was best fitted by the sum of two exponential functions, \(I_{\text{to}} = A\exp(-t/t_{\text{fast}}) + B\exp(-t/t_{\text{slow}})\), \(A+B = 1\), where \(A\) and \(B\) are amplitude terms, \(t\) is time, \(t_{\text{fast}}\) and \(t_{\text{slow}}\) are time constants of the fast and slow inactivation phases, and \(C\) is the amplitude of the steady-state component. The extent of inactivation was quantified by measuring the fraction of inactivation of the outward K\(^+\) current defined as \(I_{\text{to}}/I_{\text{to},c}\).\(^{27}\)

An expanded Materials and Methods section is available online at http://www.circresaha.org.

**Results**

**CaMKII Inhibitor KN-93 Accelerates Inactivation of Outward K\(^+\) Current \(I_{\text{to}}\)**

In the majority of human atrial myocytes studied, the outward K\(^+\) current elicited by 10-mV incremental test pulses from −60 to +60 mV was characterized by a rapidly inactivating component (\(I_{\text{f}}\)) and a sustained (\(I_{\text{ss}}\)) component (Figure 1A). External application of KN-93 (Figure 1B), a selective inhibitor of CaMKII,\(^{28}\) inhibited \(I_{\text{ss}}\) at +60 mV, 4.9 ± 0.4 pA/pF versus 2.8 ± 0.4 pA/pF, in control and in KN-93 conditions, respectively; \(n = 35\), \(P < 0.0001\); Figure 1C) and increased \(I_{\text{f}}\) (at +60 mV, 6.5 ± 0.5 pA/pF versus 7.3 ± 0.5 pA/pF; in control and KN-93 conditions, respectively; \(n = 35\), \(P < 0.01\); Figure 1D), resulting in increased extent of inactivation (\(I_{\text{to}}/I_{\text{to},c}\), see Materials and Methods) of the outward K\(^+\) current at +60 mV, \(I_{\text{to}}/I_{\text{to},c}\) 0.57 ± 0.02 versus 0.74 ± 0.02, in control and KN-93 conditions, respectively; \(n = 35\), \(P < 0.0001\)). The \(I-V\) relationships showed that \(I_{\text{ss}}\) inhibition was significant at all potentials above +10 mV and increased with depolarization, resulting in an apparent inward rectification of \(I_{\text{ss}}\) (Figure 1C). In contrast, KN-93 enhanced \(I_{\text{f}}\) at all potentials at which this current activated and shifted its voltage dependence toward negative potentials (Figure 1D). Plotting the fast inactivation time constant (\(\tau_{\text{fast}}\), see Materials and Methods) against the test voltage showed \(\tau_{\text{fast}}\) to be voltage dependent, with a mean of 188.6 ± 19.6 ms at 0 mV and 66.9 ± 4.4 ms at +60 mV (\(n = 35\)) in control conditions (Figure 1E). Application of KN-93 decreased \(\tau_{\text{fast}}\) at voltages between +20 and +60 mV (at +60 mV, \(\tau_{\text{fast}}\): 43.0 ± 4.4 ms; \(n = 35\), \(P < 0.0001\); Figure 1E) and reduced its voltage dependence.

KN-93 acted slowly, as illustrated by Figure 2A, which shows that the effects of KN-93 only started to occur after \(\sim\)3 minutes of drug exposure, whereas steady state was obtained in \(\sim\)9 minutes. Washout of KN-93 (Figure 2A) was assoc...
ated with a slow increase in $I_t$ (which reached a higher amplitude than before drug application) and with a slight recovery of $I_{sus}$. These slow changes in $I_t$ associated with KN-93 were not caused by prolonged cell dialysis or repeated membrane depolarization, as the same protocol executed with the control external solution did not cause significant changes in the characteristics of the current (not shown). Moreover, a 30-minute preincubation of rested myocytes with 20 μmol/L KN-93 also reduced $I_{sus}$ (at $160 \text{ mV}$, $2.2 \pm 0.2 \text{ pA/pF}$, n=24; Figure 2B) and accelerated the rate of $I_{t}$ inactivation (at $160 \text{ mV}$, $\tau_{fast}$: $48.8 \pm 2.8 \text{ ms}$, n=24), effects similar to those of short-term application of 20 μmol/L KN-93 on $I_{to}$. These results indicate that prolonged external application of the CaMKII inhibitor KN-93 accelerated $I_{to}$ inactivation.

**Effects of KN-93 on $I_{to}$ Are Largely due to Inhibition of CaMKII**

We examined next whether the effects of KN-93 on $I_{to}$ were related to the inhibition of CaMKII activity or to a direct effect on K$^+$ channels. We first tested the effects of the functionally inactive KN-93 analog KN-92. At a concentration of 20 μmol/L, KN-92 had no significant effect on $I_{to}$ (Figure 3B) compared with the outward K$^+$ current elicited in control conditions (Figure 3A). A higher concentration of KN-92 (100 μmol/L) significantly inhibited $I_{to}$, an effect that predominated on $I_{sus}$ (at $+60 \text{ mV}$, $30.8 \pm 14.7\%$, n=8; Figure 3C), but no significant changes in the extent of inactivation were observed. Moreover, the onset of the inhibitory effect on $I_{sus}$ during KN-92 exposure was rapid, being observed after the first pulse following KN-92 application; this contrasted with the slowly developing effect of KN-93 on $I_{to}$, which was still observed in myocytes pretreated with KN-92 (Figure 3D).

KN-93 inhibits CaMKII activity by blocking the binding of calmodulin to CaMKII, which is required for both the activation and the autophosphorylation of the enzyme.28,29 To confirm that KN-93 modulated $I_{to}$ by inhibiting CaMKII, the effects of KN-93 were studied in myocytes pretreated with the calmodulin inhibitor calmidazolium. Figure 4A shows currents recorded in control conditions and during external perfusion of 50 μmol/L calmidazolium, which inhibited $I_{sus}$ (at $+60 \text{ mV}$, $4.6 \pm 0.7 \text{ pA/pF}$ versus $3.7 \pm 0.7 \text{ pA/pF}$ in control conditions and on external application of calmidazolium, respectively; n=14, $P<0.01$; Figure 4B) and increased the extent of inactivation of $I_{to}$ (at $+60 \text{ mV}$, $I_{t}/I_{to}$: $0.60 \pm 0.03$ versus $0.67 \pm 0.03$, in control conditions and on external application of calmidazolium, respectively; n=14, $P<0.001$). Moreover, external application of 20 μmol/L KN-93 when the steady-state effect of calmidazolium had been achieved affected neither the amplitude of the outward K$^+$ current (Figure 4A) nor the $I_{sus}$ density (at $+60 \text{ mV}$, $3.4 \pm 0.7 \text{ pA/pF}$; n=14, not significant [NS]; Figure 4B); the extent of inactivation remained unchanged (at $+60 \text{ mV}$, $I_{t}/I_{to}$: $0.68 \pm 0.03$, n=14, NS).
Further evidence that $I_{to}$ is regulated by CaMKII was obtained by dialyzing cells with a peptide corresponding to CaMKII residues 281 to 309, which is a potent calmodulin antagonist containing the calmodulin binding site of CaMKII (amino acids 290 to 309) and the autophosphorylation site (Thr 286). An example of the results is given in Figure 4C, which shows the superimposition of current traces elicited by test pulses from $-60$ to $+50$ mV, recorded just after breaking the patch and after various times of intracellular dialysis with a solution containing 75 $\mu$mol/L of the peptide. This procedure was associated with a slow fall in $I_{to}$ amplitude and an increase in the extent of current inactivation. In myocytes loaded with the peptide, external application of KN-93 had additional effects on $I_{to}$ (Figure 4C), suggesting that CaMKII were only partially inhibited by the peptide. Furthermore, a sizable change in the characteristics of $I_{to}$ was observed in only 5 of the 15 myocytes dialyzed with the peptide. Neither the apparently small effect nor the low success rate of experiments with peptide 281 to 309 was due to peptide lability, as AIP, whose binding capacity cannot be altered by possible phosphorylation of Thr$^{286}$ of CaMKII. An example of the results is given in Figure 4C, which shows the superimposition of current traces elicited by test pulses from $-60$ to $+50$ mV, recorded just after breaking the patch and after various times of intracellular dialysis with a solution containing 75 $\mu$mol/L of the peptide. This procedure was associated with a slow fall in $I_{to}$ amplitude and an increase in the extent of current inactivation. In myocytes loaded with the peptide, external application of KN-93 had additional effects on $I_{to}$ (Figure 4C), suggesting that CaMKII were only partially inhibited by the peptide. Furthermore, a sizable change in the characteristics of $I_{to}$ was observed in only 5 of the 15 myocytes dialyzed with the peptide. Neither the apparently small effect nor the low success rate of experiments with peptide 281 to 309 was due to peptide lability, as AIP, whose binding capacity cannot be altered by possible phosphorylation of Thr$^{286}$-like peptide.
Inhibition of Protein Phosphatases Slows the Inactivation Kinetics of \(I_{\text{to}}\)

The effects of CaMKII inhibition on \(I_{\text{to}}\) suggested that K\(^+\) channels carrying the outward K\(^+\) current were in a phosphorylated state that could be controlled by a balance between kinase and phosphatase activities. This was tested in the next set of experiments, by studying the effect of the multifunctional phosphatase inhibitor okadaic acid on \(I_{\text{to}}\). Myocytes isolated from the same right atrial samples were separated in a group of cells treated with 500 nmol/L okadaic acid for 30 minutes before starting the experiments (n = 22) and a group of control cells (n = 20). Figure 5A shows examples of current traces recorded in myocytes from the two groups. In okadaic acid–treated cells, the outward K\(^+\) current was characterized by a slight enhancement of \(I_{\text{to}}\) (Figure 5B) and a lower density of \(I_{\text{to}}\) at +50 mV, 6.9±0.6 pA/pF versus 5.3±0.5 pA/pF, in control and okadaic acid conditions, respectively, \(P<0.05\); Figure 5C). The extent of inactivation of \(I_{\text{to}}\) was significantly decreased in the group of cells preincubated with okadaic acid compared with the control cells (at +60 mV, \(I_{\text{to}}/I_{\text{to}}\) control: 0.65±0.02 versus 0.5±0.03, \(P<0.01\)). The density-voltage relationships of the two components showed that okadaic acid, in addition to its inhibitory effect on \(I_{\text{to}}\), shifted \(I_{\text{to}}\) voltage dependence toward positive potentials (Figure 5C). Short-term external application of 500 nmol/L okadaic acid had no significant effects on \(I_{\text{to}}\).

In cells preincubated with 500 nmol/L okadaic acid (OA+), external application of 20 μmol/L KN-93 tended to inhibit \(I_{\text{to}}\) (at +60 mV, 5.7±0.7 pA/pF versus 4.1±1.1 pA/pF, with okadaic acid alone and after the addition of KN-93, respectively; Figure 5D) and decreased \(\tau_{\text{Fast}}\) (at +60 mV, 21.1±8.5%). However, the magnitude of the effect of KN-93 on \(I_{\text{to}}\) was significantly smaller in okadaic acid–treated cells (OA+) than in control cells (OA−) (Figure 5D and 5E). These results, which further demonstrate that the effects of KN-93 on \(I_{\text{to}}\) are due largely to the modulation of CaMKII activity, indicate that a balance between phosphatases and kinases regulates K\(^+\) channels in human atrial myocytes.

Modulation of \(I_{\text{to}}\) by Changes in [Ca\(^{2+}\)]

The preceding results indicating a coupling between \(I_{\text{to}}\) and CaMKII prompted us to examine whether changes in [Ca\(^{2+}\)], regulate the amplitude and time course of the transient outward current. This question was addressed first by using an internal solution containing ≈30 nmol/L of free Ca\(^{2+}\) (see Materials and Methods), a concentration that has been reported to modulate the inactivation of Kv1.4 channels. Figure 6A shows an example of the effects on \(I_{\text{to}}\) of dialyzing a myocyte with a Ca\(^{2+}\)-containing internal solution that was associated with an enhancement of the amplitude of both \(I_{\text{to}}\) (at +50 mV, +28.0±3.2%, \(n=25, P<0.0001\)) and \(I_{\text{on}}\) (at +50 mV, +5.1±1.7%, \(n=25, P<0.01\)). Furthermore, in myocytes dialyzed with a Ca\(^{2+}\)-containing internal solution, KN-93 had a marked effect on \(I_{\text{to}}\) (Figure 6D and 6E). To test whether the magnitude of the effect of [Ca\(^{2+}\)], on \(I_{\text{to}}\) depends on the basal activity of CaMKII, in another set of experiments, myocytes were incubated with KN-93 (20 to 40 μmol/L) for at least 30 minutes, and currents were recorded using a control external solution without the CaMKII inhibitor. Figure 6B shows an example of currents recorded in a myocyte pretreated with
KN-93. Dialysis of the cell with [Ca\textsuperscript{2+}]-containing internal solution caused a large increase in $I_{\text{m}}$, resulting in the apparent reversion of the effects of KN-93 on the current, particularly evident using 40 \mu mol/L KN-93. Statistical analysis confirmed that the effects of increasing [Ca\textsuperscript{2+}], on $I_{\text{m}}$ were higher in KN-93–treated than in control myocytes (at +50 mV, $I_{\text{m}}$: +35.3±4.9%, $n=10$, $P<0.001$ and $I_{\text{m}}$: +80.0±25.4%, $n=10$, $P<0.01$). As control experiments, KN-93–pretreated myocytes were dialyzed with a control internal solution, which did not cause significant changes in $I_{\text{m}}$ ($n=5$, data not shown).

In another set of experiments, the fast Ca\textsuperscript{2+} buffer BAPTA was used instead of EGTA in an attempt to reduce [Ca\textsuperscript{2+}], more efficiently in the vicinity of the channels. Intracellular application of BAPTA caused an increase in the extent of current inactivation (at +60 mV, $I/\text{I}_{\text{m}}$: 0.57±0.03 versus 0.67±0.04, 11 of 14 myocytes; $P<0.05$), as illustrated by Figure 6C. Furthermore, in BAPTA-loaded myocytes, the effects of KN-93 on $I_{\text{m}}$ and on the extent of inactivation were attenuated compared with controls (at +60 mV, $I_{\text{m}}$: 17.7±10.7%, $n=7$, $P<0.05$ and $I/\text{I}_{\text{m}}$: +0.5±10.0%, $n=7$, $P<0.01$; Figure 6D and 6E). Of note, the effects of calmidazolium on $I_{\text{m}}$ were also reduced in cells loaded with BAPTA (data not shown, $n=3$). Taken together, these results indicate that $I_{\text{m}}$ is modulated by changes in [Ca\textsuperscript{2+}], probably via CaMKII.

$I_{\text{m}}$ Is Regulated by CaMKII in Myocytes From Fibrillating Atria

The outward K\textsuperscript{+} current is altered in myocytes isolated from patients with dilated or fibrillating atria, with a more pronounced decrease in the density of $I_{\text{m}}$ than $I_{\text{m}}$, resulting in an outward K\textsuperscript{+} current with a small inactivating component. Figure 7A shows a typical example of $I_{\text{m}}$ recorded in myocytes from chronically fibrillating atria; note the prominent $I_{\text{m}}$ (at +60 mV, 4.5±0.4 pA/pF, $n=31$; NS) and reduced $I_{\text{m}}$ (at +60 mV, 4.7±0.4 pA/pF, $n=31$; $P<0.01$). In these myocytes, KN-93 accelerated the rate of the outward K\textsuperscript{+} current inactivation (Figure 7B), resulting in an almost total suppression of the maintained current (at +60 mV, 1.7±0.2 pA/pF, $n=15$; $P<0.0001$) and restoration of a large inactivating component (at +60 mV, 6.4±0.8 pA/pF, $n=15$, $P<0.001$; Figure 7C). These results indicate that CaMKII are present in myocytes from fibrillating atria and are functionally coupled to K\textsuperscript{+} channels carrying $I_{\text{m}}$.

Increased CaMKII Expression in Fibrillating Atria

To analyze the level of CaMKII expression in human atrial myocardium, Western blot analysis was performed on pro-
teins prepared from right atrial myocardium samples obtained with the same procedure as that used for the electrophysiological study. Figure 8A shows the Western blot obtained with the δ-CaMKII–specific antibody in atrial samples from patients listed in the Table. δ-CaMKII was detected in all the samples, but densitometric analysis showed that its expression was significantly enhanced from 5.9±1.0 OD (n=7) in control atria to 11.2±2.1 OD (n=5) in chronically fibrillating atria (Figure 8B; P<0.032). Immunocytochemical analysis of tissue sections with the same δ-CaMKII–specific antibody showed that specific staining predominated in atrial myocytes. The δ-CaMKII appeared to be located throughout the cell body, but more intense staining was observed in intercalated disks (Figure 9A), which contain most Kv1.5 channels.7 As a negative control, the primary antibody was preincubated with an excess of antigen, leading to the absence of specific staining (Figure 9B). A similar expression pattern was observed in tissue sections obtained from chronically fibrillating atria.

The conclusion that CaMKII regulate Ito is based on a strong body of evidence. KN-93, a specific inhibitor of CaMKII, but not its functionally inactive analog KN-92, had a marked effect on Ito. At a high concentration (100 μmol/L), KN-92 caused a use-dependent inhibition of Ito, pointing to direct binding of this molecule to K+ channels, an effect that may also be shared by KN-93 and may explain the increase in Ito after drug washout (Figure 2A). In addition, in keeping with the mechanism of action of KN-93 (which blocks the calmodulin binding to

**Clinical Characteristics of the Patients**

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</table>

AF indicates chronic atrial fibrillation; SR, sinus rhythm; AS, aortic stenosis; CI, coronary insufficiency; MI, mitral insufficiency; and TI, tricuspid insufficiency.
CaMKII),28,29 the calmodulin inhibitor calmidazolium suppressed the effects of KN-93 on \( I_{\text{to}} \). Calmodulin inhibition was associated with changes in \( I_{\text{to}} \) similar to those observed with the CaMKII inhibitor, suggesting that the two compounds modulate a common regulatory pathway. Intracellular dialysis of a synthetic peptide inhibitor of CaMKII containing the calmodulin binding site (amino acids 290 to 309) and the autophosphorylation site (Thr\(^{286}\)) of CaMKII or with AIP, a more stable peptide than the former, had effects on \( I_{\text{to}} \) similar to those of external KN-93 application, ie, an increased extent and accelerated rate of inactivation of \( I_{\text{to}} \). The low success rate in experiments with both peptides, as well as their weaker effects on \( I_{\text{to}} \) relative to those of KN-93, were likely due to the difficulty in dialyzing the subsarcolemmal region of the cells with such large molecules.32 Moreover, CaMKII may be tightly associated with K\(^+\) channels, as is the case for the N-methyl-D-aspartate receptor,33 explaining the poor accessibility of the enzymes. The finding that phosphatase inhibition by okadaic acid altered the extent of inactivation of \( I_{\text{to}} \) also indicates that the activity of K\(^+\) channels carrying \( I_{\text{to}} \) depends on their phosphorylation state. Given that pretreatment of myocytes with okadaic acid attenuated the effects of KN-93 on \( I_{\text{to}} \), CaMKII probably contribute to the tonic phosphorylation of K\(^+\) channels. Finally, cell dialysis with BAPTA, which buffers Ca\(^{2+}\) in the subsarcolemmatal space more efficiently than EGTA, also modified the rate and extent of \( I_{\text{to}} \) inactivation, indicating that the time course of the current is controlled by [Ca\(^{2+}\)]\textsubscript{i}-dependent processes. Because (1) the effects of BAPTA on \( I_{\text{to}} \) resemble those of KN-93, calmidazolium, or CaMKII inhibitory peptides and (2) the sensitivity of \( I_{\text{to}} \) for KN-93 is reduced in myocytes dialyzed with BAPTA, the effects of changes in [Ca\(^{2+}\)]\textsubscript{i}, on \( I_{\text{to}} \) may be largely mediated by CaMKII. The observation that increasing [Ca\(^{2+}\)]\textsubscript{i}, had a limited effect on \( I_{\text{to}} \), which in these conditions became exquisitely sensitive to KN-93, suggests that in human atrial myocytes and/or in our experimental conditions, CaMKII may already be activated. Indeed, pretreating myocytes with KN-93 to inhibit CaMKII enhances the effects of increasing [Ca\(^{2+}\)]\textsubscript{i}, on \( I_{\text{to}} \). The most likely explanation for this finding is that increasing [Ca\(^{2+}\)]\textsubscript{i}, causes an excess in calmodulin, which is able to recruit and activate CaMKII, probably in a competitive fashion against KN-93 in keeping with the mechanism of action of this compound.28

Although the present results point to Ca\(^{2+}\)-dependent regulation of \( I_{\text{to}} \), mainly via CaMKII activation, they do not rule out the possibility that part of the effects of KN-93 on the current are due to direct effects of the compound on K\(^+\) channels, distinct from those shared with its inactive analog KN-92. For instance, in rabbit ventricular myocytes, the peak transient outward current is blocked significantly by KN-93 but not by the inactive analog KN-92 or by a CaMKII inhibitory peptide.34 In human atrial myocytes, such direct blockade of \( I_{\text{to}} \) by KN-93 could explain the additional effect of the compound in other experimental conditions in which CaMKII was inhibited (Figures 4 and 6).

Inhibition of CaMKII markedly accelerated the rate of current inactivation, resulting in a prominent \( I_{\text{t}} \) with a shift toward negative potentials in its density-voltage relationships and a reduced \( I_{\text{rev}} \) associated with inward rectification. These effects suggest that CaMKII inhibition alters the gating characteristics of channels carrying the outward K\(^+\) current, resulting in an increased fraction of current that inactivates. Voltage-gated K\(^+\) channels, which are thought to carry the outward K\(^+\) current in cardiac myocytes, inactivate via two mechanisms: rapid N-type inactivation, which is described by a “ball-and-chain” model, and slow C-type inactivation.35 Both mechanisms are modulated by several factors,36 including serine/threonine phosphorylation processes.37 The presence of consensus sites for CaMKII38 on deduced amino acid sequences of Kv1.5 channels,\(^{9}\) the main molecular basis for \( I_{\text{to}} \), in human atrial myocytes,\(^{4,5}\) is consistent with the possibility of direct phosphorylation of these channels by CaMKII, which may modulate its rate of inactivation.31 The inactivation of K\(^+\) channels can also be markedly accelerated by coexpression of auxiliary cytoplasmic B subunits with pore-forming \( \alpha \) subunits of Kv1 channels, conferring rapid inactivation to noninactivating delayed rectifier currents.39,40 Interestingly, the interaction between \( \alpha \) and B subunits of Kv channels is also regulated by second messengers, including cAMP-dependent protein kinases (PKA) and protein kinases C, which modulate the extent of \( \alpha_{\text{F}} \)-current inactivation.27,41 It has been reported that PKA also alter Kv\(\beta_{1.3}\) subunit-mediated inactivation of Kv1.5 channels, resulting in a current with a reduced extent and rate of inactivation.42 Our results do not allow us to draw firm conclusions on the mechanism by which CaMKII regulate the inactivation of K\(^+\) channels carrying \( I_{\text{to}} \). Nevertheless, it is interesting to note that the effects of CaMKII inhibition on \( I_{\text{to}} \) share certain features with those of hKv\(\beta_{1.3}\) subunits on hKv1.5 K\(^+\) channels, which are expressed in human atrial myocardium, ie, partial inactivation and inward rectification with depolarization\(^{30}\), as with PKA,42 the interaction between the two subunits may be regulated by CaMKII.

It is already known that CaMKII regulate the inactivation of K\(^+\) channels carrying voltage-dependent outward K\(^+\) current in neurons,\(^{13}\) photoreceptor cells,\(^{14}\) and murine colonic myocytes.\(^{15}\) Indeed, the frequency-dependent inactivation of the K\(^+\) current carried by Shaker Kv\textsubscript{1.4} is regulated by CaMKII in a manner somewhat similar to the effects of these kinases on the outward K\(^+\) current of human atrial myocytes.\(^{13}\) In this latter study, increasing the [Ca\(^{2+}\)]\textsubscript{i}, or inhibiting phosphatases with okadaic acid drastically slowed the inactivation of the Kv1.4 current, which was accelerated when CaMKII were inhibited by KN-93. Taken together, these studies suggest that CaMKII are involved in controlling repolarization in excitable cells. In human atrial myocytes, the fall in the rate of \( I_{\text{to}} \) inactivation caused by CaMKII should enhance the maintained level of the outward K\(^+\) current within a large range of potentials and thus shorten the plateau phase of the action potential. As a result, Ca\(^{2+}\) influx through L-type Ca\(^{2+}\) channels and, in turn, Ca\(^{2+}\) release from the sarcoplasmic reticulum could
be reduced, thereby preventing [Ca\textsuperscript{2+}], accumulation and further activation of CaMKII. Our observation, in chronically fibrillating atrial myocardium, of upregulated expression of \(\delta\text{-CaMKII} \), which appears to be functional and coupled to K\textsuperscript{+} channels, raises questions as to the contribution of this regulatory process to the electrical remodeling that occurs during atrial fibrillation. Indeed, there is evidence that changes in [Ca\textsuperscript{2+}], homeostasis may initiate electrical remodeling during atrial fibrillation, which is characterized by a marked shortening of the action potential plateau phase.\textsuperscript{43–45} It is tempting to speculate that the upregulation of CaMKII during atrial fibrillation, by reducing the extent of inactivation of \(I_{na} \), reduces Ca\textsuperscript{2+} influx and therefore minimizes Ca\textsuperscript{2+} overload. As CaMKII are sensitive to the rate of [Ca\textsuperscript{2+}], oscillations that they can decode into distinct amounts of kinase activity,\textsuperscript{46} it is also possible that the coupling between CaMKII and K\textsuperscript{+} channels may contribute to the adaptation of the electrical activity of human atrial myocardium to sustained changes in heart rate, such as those occurring during chronic atrial arrhythmia.

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References


Regulation of the Transient Outward K⁺ Current by Ca²⁺/Calmodulin-Dependent Protein Kinases II in Human Atrial Myocytes
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